EXHIBIT E

***d*-N-Succinyl-1-methylamino-2,4-dimethyl-6-nitrobenzene.**—The less-soluble salt was decomposed with 0.5% hydrochloric acid in the same manner as the salt of the 6-bromo compound. Before crystallization from benzene the specific rotation was determined.

Rotation. (*d*-acid) 0.05 g. made up to 10 ml. with ethanol at 25° gave α_D +0.125°; *l*, 1; $[\alpha]^{25}D$ +25°.

Crystallization from benzene produced light yellow crystals; m. p. 130–131° (cor.).

Anal. (*d*-acid) Calcd. for $C_{13}H_{16}N_2O_5$: C, 55.71; H, 5.71; N, 10.00. Found: C, 56.22; H, 5.87; N, 10.07. *Rotation.* (*d*-acid) 0.05 g. made up to 10 ml. with ethanol at 25° gave α_D +0.05°; *l*, 1; $[\alpha]^{25}D$ +10°.

Racemization of *d*-N-Succinyl-1-methylamino-2,4-dimethyl-6-nitrobenzene.—The *d*-acid was completely racemized in a half hour in boiling *n*-butanol.

A solution of 0.25 g. of the *d*-acid made up to 15 ml. with methyl acetate was racemized using the technique described for the racemization of *d*-N-succinyl-1-methylamino-2-methyl-4,6-dibromobenzene. The following α_D values were obtained: at the start, +0.215°; after fifteen minutes, +0.164°; after thirty minutes, +0.121°; after forty-five minutes, +0.091°; after sixty minutes, +0.067°; after ninety minutes, +0.037°. Calculated for a reversible unimolecular reaction, the half-life was thirty-six minutes. A check racemization gave a half-life of thirty-eight minutes.

Catalytic Hydrogenation of *d*-N-Succinyl-1-methylamino-2,4-dimethyl-6-nitrobenzene; 1,5,7-Trimethylbenzimidazole-2-β-propionic Acid.—A solution of 0.25 g. of *d*-N-succinyl-1-methylamino-2,4-dimethyl-6-nitrobenzene in 125 ml. of ether was hydrogenated at room temperature and 40 lb. pressure with 0.1 g. of platinum oxide as catalyst. At the end of the reduction, the catalyst was removed by filtration and the ether evaporated by directing a stream of air onto the solution. The residue was dried in a vacuum desiccator.

No quantitative specific rotation was determined for this crude product. However, a qualitative reading showed the material to be *dextro*-rotatory.

When a solution of the crude product in *n*-butanol was refluxed for about an hour, and cooled, white crystals separated. Recrystallized from ethanol, the material melted at 265–267° (cor.). It was soluble in 5% aqueous sodium bicarbonate and was optically inactive.

Anal. Calcd. for $C_{13}H_{16}N_2O_3$: C, 67.24; H, 6.89; N, 12.06. Found: C, 67.22; H, 6.82; N, 12.06.

Summary

1. Several new N-succinyl-1-methylamino-2,4-dimethyl-6-substituted benzenes and N-succinyl-1-methylamino-2-methyl-4,6-dibromobenzene have been prepared, resolved and the half-lives of the optically active forms determined.

2. The half-lives of the series are as follows: bromine, three and one-tenth hours; iodine, twenty and one-half hours; nitro, six-tenths hour; and the dibromo compound, one and one-tenth hours. Boiling *n*-butanol was used as solvent except in the case of the nitro compound where boiling methyl acetate was employed. The corresponding methyl and methoxyl analogs have been prepared previously and their half-lives are nine hours in boiling *n*-butanol and two and seven-tenths hours in boiling methyl acetate, respectively.

3. From these values, after comparison with the interference effects produced by the same groups in the biphenyl series, it was concluded that factors other than size of the groups were influencing the rates of racemization.

4. The basicity of aryl amines is reduced by the substitution of electronegative groups in the *o*-, *m*- or *p*-positions. A decrease in the expected stability of the optically active amines is observed with similar substituents. From these facts it appears that the increased double-bond character of the carbon–nitrogen bond in these amines of decreased basicity facilitates racemization. The tendency to form a double bond will aid in forcing the substituents on the amino nitrogen into a coplanar configuration with the ring.

URBANA, ILLINOIS RECEIVED MARCH 11, 1948

[CONTRIBUTION FROM THE WESTERN REGIONAL RESEARCH LABORATORY[1]]

The Reaction of Formaldehyde with Proteins. V. Cross-linking between Amino and Primary Amide or Guanidyl Groups

BY HEINZ FRAENKEL-CONRAT AND HAROLD S. OLCOTT

According to the opinion of most experts in the field, the tanning or hardening action of formaldehyde is probably not due to its primary addition to the amino or any other type of protein group, but rather to a secondary condensation reaction which transforms the methylol (—CH_2OH) groups into cross-linking methylene (—CH_2—) bridges. The first experimental evidence for the occurrence of condensation was supplied by Nitschmann and his co-workers, who showed that there was a loss of water during the reaction of casein with gaseous formaldehyde.[2,3] Proof for cross-linking was obtained in studies from this Laboratory, in which it was demonstrated that the average molecular weights of salmine[4] and other proteins[5] could be increased by formaldehyde treatment.

The question arose: Which protein group or groups are available for such condensation reactions with formaldehyde? Nitschmann, *et al.*,[2] showed that the amino groups were directly concerned.[6] Thus casein, in which the amino groups were largely protected with acetyl groups, bound formaldehyde only by addition, while condensation occurred when unmodified casein was used. However, experiments with salmine,[4] which con-

(1) Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, U. S. Department of Agriculture. Article not copyrighted.

(2) Nitschmann and Hadorn, *Helv. Chim. Acta*, **27**, 299 (1944).

(3) Nitschmann and Lauener, *ibid.*, **29**, 174 (1946).

(4) Fraenkel-Conrat and Olcott, THIS JOURNAL, **68**, 34 (1946).

(5) Mecham and Fraenkel-Conrat, in preparation.

(6) See also Gustavson, *J. Int. Soc. Leather Trades Chem.*, **24**, 377 (1940); *Kolloid Z.*, **103**, 43 (1943); *J. Biol. Chem.*, **169**, 531 (1947).

tains no primary amino groups,[7] suggested that at 70° guanidyl groups also react with formaldehyde to form cross-linking methylene groups. The isolation of djenkolic acid ($HOOC-CHNH_2-CH_2-S-CH_2-S-CH_2-CHNH_2-COOH$) from reduced keratins that had been treated with formaldehyde at elevated temperature[8] indicated that, under these conditions, cross-linking occurred between sulfhydryl groups. Nitschmann and Hadorn[2] proposed that the formation of methylene bridges between amino and peptide groups accounts for the tanning action of formaldehyde but this was not proved. Reactions involving pairs of amino[6] or of peptide groups[9] have also been hypothesized but appear to be chemically improbable. On the basis of experiments with formaldehyde and casein or casein derivatives, Wormell and Kaye[10] proposed the hypothesis that acid-hardening of this protein "occurs largely by means of cross-linking on to the amide groups using the formaldehyde already attached to amino groups."

It will be shown in the present study that, at room temperature and over a wide *p*H range, formaldehyde can form cross-linking methylene bridges between *amino* groups on the one hand, and *primary amide* or *guanidyl* groups on the other. The

$$R-NH_2 + HCHO + H_2NCO-R \longrightarrow R-NH-CH_2-NHCOR + H_2O \quad (I)$$

$$R-NH_2 + HCHO + NH_2-C(=NH)-NHR \longrightarrow R-NH-CH_2-NH-C(=NH)-NHR + H_2O \quad (II)$$

secondary amide groups of peptide bonds do not participate in Reaction I at room temperature. It will further be shown that, by the same reactions, ammonia or simple primary amines can be bound to protein amide groups; and simple amides or guanidines to the protein amino groups. The data suggest that the tanning action of formaldehyde on proteins at room temperature may be due largely to Reactions I and II.

Condensation products of low molecular weight secondary amines, formaldehyde and primary amides were described by Einhorn[11] but the reaction apparently has not since been studied in detail nor have its implications for the tanning mechanism been previously recognized.

Results

A. Model Experiments.—Model experiments with simple compounds were helpful in ascertaining which types of protein groups might be expected to become cross-linked through methylene bridges. For most of these experiments, 0.4–0.5 molar solutions of two different compounds were treated with about 2 molar formaldehyde at room temperature and at a *p*H where each compound by itself bound little if any formaldehyde in a manner which was not reversed in the presence of dimedon at *p*H 4.6. The binding of appreciable amounts, under such conditions, was regarded as evidence for a condensation reaction involving both nitrogenous components of the system. This conclusion was usually corroborated by other means of analysis and, in some cases, by isolation of the reaction products.

The limitations of such an experimental approach are two-fold: (1) Results obtained with compounds which by themselves bind formaldehyde over a wide *p*H range, *e. g.*, guanidines,[12] cannot be unequivocally interpreted as evidence for cross-linking. (2) Some methylene compounds may be so unstable as to yield their formaldehyde in the presence of dimedon.[13]

With amides and most amines, however, these limitations do not apply. Pairs formed by a primary amide, on the one hand, and a primary or secondary amine or an amino acid on the other, give condensation products with formaldehyde over the range of *p*H 3.2 to 7.6 (Reaction I) (Tables I and II).[14] More alkaline or more acid solutions could not be studied in this manner, since they catalyze the stable fixation of increasing amounts of formaldehyde by the amides alone[12]

(7) Fraenkel-Conrat and Olcott, *Fed. Proc.*, **6**, 253 (1947).

(8) Consden, Gordon, and Martin, *Biochem. J.*, **40**, 580 (1946); Middlebrook and Phillips, *ibid.*, **41**, 218 (1947).

(9) Küntzel, *Angew. Chem.*, **50**, 309 (1937).

(10) Wormell and Kaye, *J. Soc. Chem. Ind.*, **64**, 75 (1945).

(11) Einhorn, *Ann.*, **343**, 207 (1905); **361**, 113 (1908).

(12) Unpublished experiments have shown that, at room temperature, guanidines bind formaldehyde slowly over the range of *p*H 3–7, but much more rapidly above *p*H 10 and below *p*H 2; between *p*H 10 and 11 about one-half mole of formaldehyde per mole guanidine was bound in eight hours. The reactivity at elevated temperature was studied previously.[4]

(13) The apparent non-participation of sulfhydryl groups in methylene compound formation (Table XI) is possibly ascribable to such lability. A reaction involving amino and sulfhydryl groups might be assumed to occur in view of the ease with which cysteine condenses with formaldehyde to form the cyclic thiazolidine carboxylic acid [Schubert, *J. Biol. Chem.*, **111**, 671 (1935); **114**, 341 (1936); Ratner and Clarke, THIS JOURNAL, **59**, 200 (1937)].

(14) Methylamine alone, of all amines studied, bound formaldehyde at *p*H 3.0 in a manner stable to dimedon but reversible by acid hydrolysis (Table II). The fact that the cyclic trimer of methylamine and formaldehyde also did not release most of its formaldehyde in the presence of dimedon suggests that this compound may form in the solution at *p*H 3.0. This trimer differs from other amine–aldehyde reaction products, including the butylamine–formaldehyde trimer, in yielding a water-soluble stable condensation product with dimedon at *p*H 4.6. Thus, if first exposed to buffers of *p*H 3–5 for twenty-four hours at room temperature, almost the entire formaldehyde of the trimer (94%) can be precipitated with dimedon, but if dimedon and *p*H 4.6 buffer are added as usual, simultaneously, only about 10% of the expected amount of the insoluble condensation product is obtained; and upon addition of excess free formaldehyde to the solution, only about 60% of the added dimedon is precipitated, indicating some fixation in soluble form. Higher aliphatic amines were found to bind formaldehyde in an acid-irreversible manner when acetic acid or its homologs were used as buffers. ε-Aminocaproic acid showed the same phenomenon by itself (Table II). It appears probable that this may be attributed to the availability of the $-CH_2-COOH$ group for a Mannich type of condensation[15] with the amines and formaldehyde. This reaction is largely suppressed in favor of Reaction I if amides are also present. In contrast to amine acetates, amino acids and amine hydrochlorides do not condense to an appreciable extent with formaldehyde and acetic acid within three days at room temperature and *p*H 3–5 (Table II).

(15) Blicke, in "Organic Reactions," Vol. I, John Wiley and Sons, Inc., New York, N. Y., 1942.

TABLE I

CONDENSATION REACTION OF FORMALDEHYDE WITH AMINO OR IMINO ACIDS AND AMIDES[a]

Reactants	pH	Time, days	Formaldehyde bound, equivalents/basic N[b] Total	Irreversibly
Alanine + acetamide	3.5	1 2 5 12	0.61 0.67 0.64 0.72	0.0 (2 days)
Alanine	3.5	1 2 5 12	.0 0.0 0.03 0.09	
Acetamide	3.2	1 2 5 12	.05 0.10 0.20 0.40	
Alanine + acetamide	4.3	2 4	.60 0.71	.16 (4 days)
Alanine	4.3	2 4	.0 0.23	
Acetamide	4.3	2 4	.06 0.11	
Alanine + acetamide	7.6	1 7	.75 1.15	
Alanine (0.4 *M*)	7.6	1 7	.22 0.78	.04 0.40
Acetamide	7.6	1 7	.05 0.44	
Alanine + propionamide	3.5 4.0	1 1	.38 0.48	.0 (*p*H 4.0)
Propionamide	3.5 4.0	1 1	.10 0.08	
Alanine + propionamide	4.8	3 3	.42[c] 0.60	.0
Propionamide	4.8	3 3	.00[c] 0.04	.0
Glycine + acetamide	3.5	1	.67	
Glycine	3.6	1	.02	
Glycine (0.5 *M*) + acetamide (0.5 *M*)	4.7[c]	1	.67	.0
Glycine (1.0 *M*)	4.2[c]	1	.06	
Proline + acetamide[d]	7.6	1	.62	
Proline	7.6	1	.0	
Proline + acetamide	4.4	1	.30	
Proline	4.5	1	.07	
Acetamide	4.4	1	.04	
Sarcosine·HCl + acetamide	3.4	3	.90	
Sarcosine·HCl	3.4	3	.0	
Sarcosine·HCl + acetamide	4.2	3	.71	
Sarcosine·HCl	4.2	3	.0	
Sarcosine·HCl + acetamide	7.6	3	.64	
Sarcosine·HCl + aceturic acid	7.6	3	.0	
Sarcosine·HCl + benzoylalanine	7.6	3	.0	
Sarcosine·HCl	7.6	3	.0	
Threonine + acetamide	4.2	3	.24	
Threonine	4.2	3	.04	
Alanylalanine + alanine	4.2	3	.17	
Alanylalanine (0.4 *M*)	4.2	3	.15	
ε-Aminocaproic acid + acetamide	3.4	3	.64	.14
ε-Aminocaproic acid	3.4	3	.52	.38
ε-Aminocaproic acid + acetamide	5.4	1 3	.75 0.95	.24 0.24
ε-Aminocaproic acid	4.9	1 3	.62 0.66	.39 0.58
ε-Aminocaproic acid + acetamide	2.4[e]	3	.64	.06
ε-Aminocaproic acid	2.4[e]	3	.12	.06

[a] Unless otherwise stated, the amino acid or amide alone was treated in 0.8 *M* solution. Amino acid + amide solutions were 0.4 *M* in regard to each reactant; the formaldehyde, 1.5 to 2.5 *M*. Except as noted, the solutions at *p*H 3.2–4.3 were buffered with approximately 0.5 *M* acetic acid or acetate; those at *p*H 4.4–7.6 with approximately 0.6 *M* phosphate. Reactions were performed at room temperature (approximately 23°). [b] Or amide-N when no basic nitrogen was present. [c] No buffer was used. [d] For the rate curve of this reaction see Fig. 1. [e] Oxalic acid (0.3 *M*) used as buffer.

(Table III). However, indications were obtained by other techniques that a condensation reaction involving alanine and acetamide did not occur appreciably below *p*H 1 and occurred only to a small extent above *p*H 11 (Table III).

A comparison of the rate and extent of the reaction occurring at *p*H 3–4 and *p*H 6–7 indicated that with amino acids and amides the neutral medium was the more favorable (Table I, Fig. 1). With amines and amides, however, condensation occurred better in acid solution. This difference was attributed to the rapid formation and stability of cyclic trimers of the amines and formaldehyde in alkaline solution (*cf.* footnote 14) and could be demonstrated by treating such trimers with acetamide at both *p*H levels. Amine–methylene–amide formation occurred at *p*H 4 but not at *p*H 8.3 (Table IV).

As expected, the condensation reaction was favored by high concentrations of the reactants

TABLE II

CONDENSATION REACTION OF FORMALDEHYDE WITH AMINES AND AMIDES[a]

Reactants	Molarity of Amine	Amide	Formaldehyde	Buffer	Final pH	Time, days	Formaldehyde bound, equivalents/basic N[b] Total	Irreversibly
Methylamine·HCl + acetamide	0.5	0.5	2.5	None	3.8	1	0.57[c]	0.0
Methylamine·HCl	1.0	..	2.5	None	3.0	1	.24[c]	
Dimethylamine·HCl + acetamide	0.45	.45	2.5	None	4.7	1 3	.31 0.35	
Dimethylamine·HCl	.9	..	2.5	None	5.0	1 3	.06 0.06	
n-Butylamine·HCl + acetamide	.25	.25	1.25	None	4.4	1 3	.58 0.70	.0 (3d)
n-Butylamine·HCl + propionamide	.25	.25	1.25	None	4.6	1 3	.58 0.70	
n-Butylamine·HCl	.5	..	1.25	None	3.9	3	.04	
n-Butylamine·HCl	.5	..	1.25	Acetic acid[d]	3.2	3	.00	
i-Butylamine + acetamide	.4	.4	2.0	Acetic acid[d]		3	.69	.12
i-Butylamine	.8	..	2.0	Acetic acid[d]	4.4	3	.79	.56
i-Butylamine	.8	..	2.0	Formic acid	3.8	3	.58	.45
i-Butylamine	.8	..	2.0	Butyric acid	4.9	3	.92	.67
i-Butylamine·HCl	.8	..	2.0	Acetic acid	2.5	3	.10	.0
Ethanolamine·HCl + acetamide	.43	.5	2.5	None	4.4	0.2 1 3	.63 0.70 0.70	.0 (1d)
Ethanolamine·HCl	.86	..	2.5	None	4.0	0.2 1 3	.0 0.0 0.0	.0 (1d)
Ethanolamine·HCl	.60	..	1.5	Acetic acid	2.4	1	.00	
Ethanolamine + acetamide	.95	1.0	2.4	Oxalic acid		1 5	.60 0.70	.. 0.05
Ethanolamine	.95	..	2.4	Oxalic acid		1 5	.05 0.25	.. 0.15
Ethanolamine + acetamide	.95	1.0	2.4	Acetic acid		1 3	.38 0.67	.0 0.22
Ethanolamine	.95	..	2.4	Acetic acid		1 3	.25 0.65	.15 0.49
Ethanolamine + acetamide	.25	0.25	1.25	Phosphate	8.0	1	.53	
Ethanolamine	.5	..	1.25	Phosphate	8.5	1	.25	.17
Ethanolamine + acetamide	.25	.25	1.25	Phosphate	5.9	1	.55	
Ethanolamine	.5	..	1.25	Phosphate	6.3	1	.22	.20
Ethanolamine + acetamide	.25	.25	1.25	Phosphate	6.8	1	.62	
Diethanolamine·HCl + acetamide	.43	.45	2.5	None		1 3	.06 0.06	
Diethanolamine·HCl	.85	..	2.5	None		1 3	.00 0.00	
1,4-Diaminobutane·2HCl + acetamide	.4	.4	2.0	Acetic acid		3	.62	.14
1,4-Diaminobutane·2HCl	.8	..	2.0	Acetic acid		3	.20	.20
Glycine ethyl ester·HCl + acetamide	.4	.4	2.0	Acetic acid	3.0	1 3	.26	.20
Glycine ethyl ester·HCl	.8	..	2.0	Acetic acid	3.0	1 3	.00 0.02	
Glycine ethyl ester·HCl + acetamide	.5	.5	2.5	None	3.6	1	.28	
Glycine ethyl ester·HCl	.5	.5	2.5	None	3.2	1	.00	
Acetamide	..	.8	2.0	Acetate	4.3	2 4	.06 0.11	
Acetamide	..	.8	1.5	Acetic acid	3.2	2 5	.10 0.20	
Acetamide	..	.7	1.8	Oxalic acid	2.0	1	.55	.0
Propionamide	..	.8	2.0	Acetate	4.0 4.8	1	.08 0.08	
Propionamide	..	.8	2.0	Acetic acid	3.3	3	.30	.0
Propionamide	..	.7	2.0	Oxalic acid	2.0	1	.63	.0

[a] Reactions performed at room temperature. [b] Or amide-N if no basic nitrogen is present. [c] Similar results were obtained when acetic acid was also present. Concerning the fixation of formaldehyde by methylamine hydrochloride, *cf.* footnote 14. [d] Similar results were obtained with formic and butyric acid.

(Table V). It reached equilibrium within twenty-four to forty-eight hours, at which time the formaldehyde bound corresponded usually to 60–100% of that equivalent to the amine or amide used. As final proof for the occurrence of the reaction, the formaldehyde condensation products of acetamide with alanine and proline were isolated and characterized.

The non-reactivity of secondary amides (—CO—NHR) for the condensation reaction as contrasted to the primary amides (—CO—NH_2) is significant (Table I).[16,17] Cross-linking has often been postulated as involving mainly the peptide linkage. In the light of the present findings, it would appear that the peptide bonds cannot be involved in the tanning reactions occurring at room temperature.

(16) Einhorn[11] found that primary and secondary amides differed also in their ability to add formaldehyde to form methylol groups.

(17) French and Edsall, "Adv. in Protein Chemistry," Vol. II, Academic Press, Inc., New York, N. Y., 1945, p. 277.

TABLE III

EFFECT OF EXTREMES OF *p*H ON ALANINE–ACETAMIDE–FORMALDEHYDE REACTION[a]

Alanine $\times 10^{-1}$ mole	Acetamide $\times 10^{-1}$ mole	Solvent	Final *p*H[b]	Apparent amino-N[c] $\times 10^{-3}$ mole	Formaldehyde bound $\times 10^{-3}$ moles
1.0	...	0.6 ml. 6 *N* HCl	0.85	1.0	0.0
1.0	1.0	0.6 ml. 6 *N* HCl	0.85	1.15	.63
...	1.0	0.6 ml. 6 *N* HCl	0.85	0.24	.52
1.0	...	0.6 ml. 2 *N* NaOH	11.3	.83	.01
1.0	1.0	0.6 ml. 2 *N* NaOH	11.1	.70	.49
...	1.0	0.6 ml. 0.33 *N* NaOH	11.45	.16	.58
1.0	...	0.6 ml. H_2O	4.7	.94	.01
1.0	1.0	0.6 ml. H_2O	4.7	.48	.57
...	1.0	0.6 ml. H_2O	4.5	.06	.02

[a] 24 hours at room temperature. All plus 0.5 ml. 7.5% formaldehyde (1.25 mM). [b] Measured after dilution to 10 ml. [c] See experimental part for discussion.

TABLE IV

USE OF CYCLIC TRIMERS OF METHYL- AND BUTYLAMINE AND FORMALDEHYDE FOR CONDENSATION REACTION WITH AMIDE

Reaction mixture	Final *p*H	Equiv. of formald. bound[a]
1,3,5-Tri*methyl*trimethylenetriamine (0.33)[b] + acetamide (1.0)	4.0	0.5[a]
1,3,5-Tri*methyl*trimethylenetriamine (0.33)[b]	4.0	0.11
1,3,5-Tri*methyl*trimethylenetriamine (0.33)[b] + acetamide	8.3	0.07[c]
1,3,5-Tri*methyl*trimethylenetriamine (0.33)[b]	8.3	0.0[c]
1,3,5-Tri*butyl*trimethylenetriamine (0.33) + acetamide (1.0)	5.0	0.55
1,3,5-Tri*butyl*trimethylenetriamine (0.33)[b]	5.0	0.13

[a] Stably in presence of dimedon at *p*H 4.6. [b] Figures in parentheses represent millimoles used. Each reaction mixture contained also 1.5 ml. 3 *M* acetic acid (*p*H 4 and 5) or phosphate buffer (*p*H 8.3). They were held for three days at room temperature. [c] To assure complete liberation of formaldehyde from 1,3,5-tri*methyl*trimethylenetriamine an aliquot of the diluted alkaline reaction mixture was held for 1 day in the *p*H 4.6 buffer before addition of the dimedon (*cf.* footnote 14).

TABLE V

EFFECT OF CONCENTRATION OF REACTANTS ON CROSS-LINKING REACTION (A); AND LACK OF EFFECT ON ACETAMIDE–FORMALDEHYDE (B); AND ALANINE–FORMALDEHYDE REACTION (C)[a]

	Molarity of					Formaldehyde bound, equivalents/basic N[b]		
	Alanine	Acetamide	Formaldehyde	Time, days	Final *p*H			
A:	0.14	0.14	1.4	3	3.4	0.17		
	.4	.4	1.5	2	3.4	.67		
	.4	.4	0.75	2	3.4	.44		
	.4	.4	0.5	3	3.4	.36		
B:	..	.8	1.5	2 5 12	3.4	.10	.20	.40
	..	.16	1.5	2 5 15	3.4	.08	.19	.50
C:	.8	..	1.5	5	7.6	.55		
	.16	..	1.5	5	7.6	.5		

[a] Reactions performed at room temperature, with acetic acid or phosphate as buffers. Alanine itself reacts at an appreciable rate with formaldehyde only at *p*H 7 or above. [b] Or amide-N if no basic N is present.

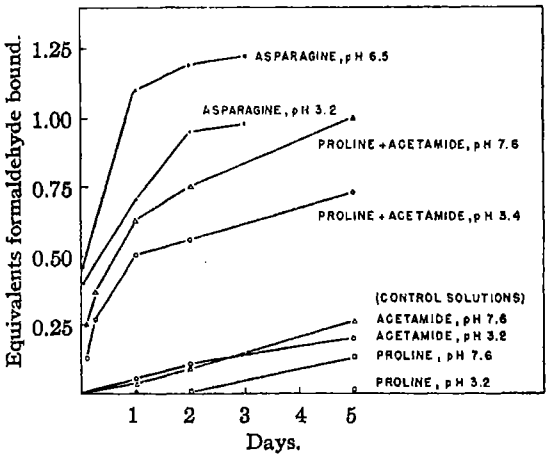

Fig. 1.—Rate of fixation of formaldehyde by various compounds at 23°: asparagine, 1 mM. in 3.5 ml. containing 2.5 mM. formaldehyde and approximately 0.5 molar acetate or phosphate buffer. For composition of other reaction mixtures, see Table I. The amount of unbound formaldehyde was determined by precipitation with dimedon.

The mechanism of Reaction (I) has not been clearly established. Like the Mannich reaction,[15] it is a condensation involving a primary or secondary base, formaldehyde, and a reactive hydrogen, furnished in this case by a primary amide group. It would appear probable that this occurs in two steps. Einhorn[11] believed that the amido-methylol compound formed first, but presented no conclusive evidence in support of this mechanism. However, addition of formaldehyde to the amide group in the absence of an amine proceeds much more slowly than the cross-linking reaction and thus probably does not represent an intermediate stage. This is further indicated by the inability of amido-methylol compounds to condense with the amines (see Experimental).

The alternate possibility is that the amine reacts first to give an aminomethylol (Reaction III) which then condenses with the amide (Reaction IV).

$$R{-}NH_2 + CH_2O \longrightarrow R{-}NHCH_2OH \quad (III)$$

$$R{-}NH{-}CH_2OH + NH_2{-}CO{-}R' \longrightarrow R{-}NH{-}CH_2{-}NH{-}CO{-}R' + H_2O \quad (IV)$$

In neutral and alkaline solution, amines are known to be transformed by formaldehyde to methylol and dimethylol compounds, but no evidence appears to be available that such reactions occur also at *p*H 3–4. Because of the lability of the amino–methylol linkage, the occurrence of this reaction cannot be demonstrated by the dimedon or Van Slyke amino nitrogen techniques.[18] That formaldehyde actually reacts with amino groups within a few hours at *p*H 3.6 or lower is indicated by (1) a fall in the *p*H of the reaction mixtures (Table VI)

(18) The *slow* decrease in the formaldehyde and amino nitrogen contents of amino acid–formaldehyde reaction mixtures[17] is evidence of secondary reactions, possibly of the Mannich type, rather than of the primary addition reaction.

TABLE VI

EFFECT OF FORMALDEHYDE ON SOLUTIONS OF AMINE HYDROCHLORIDES AT *p*H 3.6[a]

Amine	Drop of *p*H after addition of formaldehyde: Original *p*H	Final *p*H	Time until constant, min.	Equivalents of NaOH needed to titrate back to original *p*H
Methyl	3.6	1.2	120	0.170
Dimethyl	3.6	2.9	60	.0018
Butyl	3.6	2.5	60	.014
Ethanol	3.6	2.2	30	.029
Diethanol	3.6	3.1	10	.0014

[a] To 10 ml. of 1.5 *M* solutions of the amine hydrochlorides adjusted to *p*H 3.6 ± 0.1 were added 4 ml. of commercial 40% formalin (*p*H 3.6).

and (2) a change in optical rotation of solutions containing L-amino acids and formaldehyde. The rate of reaction (III), even in acid solution, appears to be sufficiently fast, compared to the cross-linking, to allow it to be the first step of the latter reaction.

While it is probable that in acid solution the equilibrium of Reaction III is greatly to the left, this may well be shifted by the occurrence of Reaction IV to permit the cross-linking to proceed to the observed extent. Changes in the optical rotation of reaction mixtures containing an optically inactive amide, an optically active amine, and formaldehyde lend independent support to the two-stage mechanism here proposed (see Experimental). The initial formation of the aminomethylol compound is also now favored as occurring during typical Mannich reactions.[15,19]

B. Experiments with Proteins or Macromolecular Model Compounds.—A second experimental approach consisted in studying the introduction, by means of formaldehyde, of simple amines, amides, or guanidines into proteins or other macromolecular materials rich in amide, amino, or guanidyl groups. The data generally support the findings obtained with the model systems. Gliadin and polyglutamine, both very rich in *amide groups*, bind in acid solution large amounts of ammonia, primary amines, or amino acids (Table VII). Imino acids appear to be bound only to a limited extent by gliadin and not above *p*H 2.0 by polyglutamine, although they were found to react readily with acetamide over a wide *p*H range (Table I). Since the resultant linkage was found to be relatively labile, the possibility exists that greater amounts of the secondary amines were actually bound by polyglutamine and gliadin than could be demonstrated after extensive periods of dialysis. It seems that in acid solution the amido–methylol linkage, though forming more slowly, is more stable than the methylene cross-links here described. This probably explains why in 50% acetic acid–formalin much more alanine is introduced into gliadin in two hours than after three days (Table VII).

When proteins or a tyrosine-formaldehyde polymer[20] rich in *amino groups* were treated with formaldehyde in the presence of acetamide, considerable amounts of this compound were introduced at room temperature over the range of *p*H 2–8 (Table VIII). As expected, this reaction did not occur appreciably, however, if most of the amino groups of the protein were blocked by acetylation.

The cross-linking of amino with guanidyl compounds by formaldehyde is demonstrated by the introduction, over the range of *p*H 4.2–8.5, of methylguanidine into proteins rich in amino groups (Table IX). On the other hand, the inability of amide and guanidyl groups to condense under similar conditions is shown with polyglutamine (Table VII) and salmine (Table VIII). Both of these latter conclusions were suggested but not conclusively demonstrated by the model experiments. The fixation of all low molecular compounds by proteins is usually accompanied by an analytically demonstrable increase in the amounts of formaldehyde bound over those bound under the same conditions in the absence of the added compounds.

A particular case of amine–amide interaction was studied in some detail, because of its special interest and possible practical importance. Swallen and Danehy[21,22] have described a remarkable catalytic effect of amines or ammonium ions on the reaction of formaldehyde with zein. When a 20% solution of zein in glacial acetic acid is treated with an equal volume of commercial formalin, a gel forms within a few minutes only if traces of ammonium ions or primary amines are added; otherwise, the mixture remains fluid for several hours.[23] Similar experiments have now been performed with gliadin. This protein also sets quickly to a gel only after the addition of small amounts (*e.g.*, 2 equivalents per 10^4 g.) of ammonia or primary amines. Commercial gliadin and a preparation made by a technique involving isoelectric fractionation from acetic acid solution behaved like zein in gelling slowly even without added amines, but gliadin carefully prepared from wheat gluten by fractionation with cold alcohol did not gel at all until an amine was added as "catalyst".[24] Gels formed in the presence of small amounts of "catalyst" were found to liquefy if the reaction mixture was held for one to three days at room temperature. In contrast permanent gels and insoluble products were obtained when greater amounts of amine had been added.

(19) Bachmann and Heisey, THIS JOURNAL, **68**, 2406 (1946).

(20) Obtained by treating tyrosine with formaldehyde in acid solution. The preparation used contained 4.8% amino nitrogen (Olcott, in preparation).

(21) Swallen, *Ind. Eng. Chem.*, **33**, 397 (1941).

(22) Swallen and Danehy, in Alexander, "Colloid Chemistry," Vol. VI, Chemical Catalog Co., New York, N. Y., 1946, p. 1140.

(23) The authors are indebted to J. P. Danehy, Corn Products Refining Co., for drawing their attention to this phenomenon.

(24) The same behavior was noted with "gliadin sulfate," the water-soluble fraction resulting from the sulfation of wheat gluten with concentrated sulfuric acid. This derivative contains acid sulfuric esters on the hydroxyl groups and sulfonic acid substituents on the phenolic groups of the protein [Reitz, Ferrel, Fraenkel-Conrat and Olcott, THIS JOURNAL, **68**, 1024 (1946)].

TABLE VII

FIXATION OF AMINES BY MACROMOLECULAR COMPOUNDS RICH IN AMIDE GROUPS THROUGH FORMALDEHYDE CONDENSATION[a]

Macromolecular compound	Additive	Final pH	Equivalents per 10^4 g. Bound additive[b]	Bound formaldehyde	Increased acid groups
Polyglutamine[c]	Ammonium chloride[d]	2.5	23	33	
	Methylamine·HCl[d]	2.6	18	17	
	Dimethylamine·HCl	3.2	0	8	
	Alanine·HCl	2.8	32	25	14[e]
	Sarcosine·HCl	2.0	10	4	3.4
	Proline·HCl	2.2	0	7	0
	Piperazine·2HCl	2.6	20	25	
	Methylguanidine·$^1/_2H_2SO_4$	3.2	0	3	
	Morpholine oxalate	4.2	20	23	
	Ammonium acetate	3.6	6	8	
	Methylamine	4.3	10	16	
	Alanine	3.3	28	24	6[e]
	Alanine	4.8	18	22	14
	Sarcosine	4.9	0	7	0
	Proline	3.3	0	3	0[e]
	Proline	4.8	0	1	0
	None	3.4	..	7	..
Gliadin	Ammonium chloride (2 hours)[g]	2.2	(22)		
	Ammonium acetate (2 hours)[g]	2.8	(9)		
	Alanine (2 hours)	2.6	(23)	15	14.6
	Alanine (3 days)	2.6	(3)		2.0
	Proline (2 hours)	2.6	(7)	6	2.3
	Proline (3 days)	2.6	(7)		2.3
	Amino ethyl sulfuric acid (7 days)	2.6	10.0[h]	21	
	None (2 hours)	2.5	..	4	..
	None (7 days)	2.5	..	20	..
Gliadin[i]	Ammonium chloride[g]	2.3	(17)		
	Ammonium acetate (30% insol.)	3.4	(7)		
	Amino ethyl sulfuric acid	2.9	9.8[h]		6.4
	Alanine	3.2	(9)		5.9
	Proline	3.2	(7)		2.0
	Alanine·HCl	2.2	(5)		2.1
	Sarcosine·HCl	2.2	(7)		0.0
	Piperazine·2HCl[g]	2.4	(9)		
	Ethylenediamine[g]	3.9	(2)		
	Ethylenediamine·2HCl[g]	2.1	(5)		

[a] Polyglutamine and gliadin contain 52 and 32 amide groups per 10^4 g., respectively. [b] Approximate values, obtained by Kjeldahl nitrogen analysis after thorough dialysis, except where otherwise noted. Nitrogen recoveries for gliadin were unreliable as a measure of the amounts of amine introduced since part of the protein became dialysable during the reaction. (Recovery in control samples consistently only 87%.) The uncertain values are in parentheses. [c] 5% solutions of the polyamide, 4% in regard to formaldehyde were treated for five days with 80 equivalents of the various amine salts or amino acids (per 10^4 g.), unless otherwise specified. The solutions contained 1.0–1.5 *M* acetic acid or acetate buffer. [d] In these cases gels formed within one and two days, respectively. When the reaction mixtures were made up five times more dilute, no gels formed, but only 2 and 0 equivalents of ammonia or methylamine were bound, respectively, and correspondingly small amounts of formaldehyde (2 and 1 equivalent). When the reactions were terminated before gelling occurred (after six and twenty-four hours, respectively), 8 and 5 equivalents of the amines were introduced, and 6 and 3 equivalents of formaldehyde. [e] Analyzed after many weeks of dialysis for the purpose of molecular weight determinations. [f] 10% gliadin in 50% glacial acetic acid–50% formalin mixture, with 32 equivalents of the additives. Reaction times indicated in parentheses. [g] Products largely insoluble. [h] Calculated from amount of sulfate sulfur introduced. [i] 4% gliadin, 8% formaldehyde, 0.6 *M* acetic acid, and 32 equivalents of the various amines were used. Reactions were allowed to proceed for four days at room temperature.

With regard to the nature of the amine, all primary amines used except α-aminoisobutyric acid, glucosamine, serine and threonine were effective. In equivalent amounts, the different amines caused gelling after varying time periods. Those acting most rapidly were: ammonia, glycine, alanine, arginine, ethanolamine, 2-aminoethylsulfuric acid and aniline. Glutamic and ε-amino-*n*-caproic acids, butylamine, hexadecylamine, semicarbazide, hydroxylamine and hydrazine acted more slowly. In contrast dimethylamine, diethanolamine, proline, hydroxyproline, sarcosine, benzoylalanine, N-mesyltyrosine, guanidine and urea did not favor gel formation. Piperazine

TABLE VIII

FIXATION OF ACETAMIDE BY MACROMOLECULAR COMPOUNDS CONTAINING AMINO OR GUANIDYL GROUPS THROUGH FORMALDEHYDE CONDENSATION[a]

Macromolecular compound	Reactive groups per 10^4 g.	Additive	Final *p*H	Equivalents bound per 10^4 g. Acetamide	Equivalents bound per 10^4 g. Formaldehyde
Bovine serum albumin (BSA)	9.7 amino	Acetamide	1.4	1.9	
		Acetamide	2.1	7.4	
		Acetamide	3.2	6.2	
		Acetamide	4.2[b]	7.0	7
		Acetamide	4.8	5.9	
		Acetamide	7.4[c]	5.7	5
		Acetamide	11.0	1.5	
		None	4.2[c]		3
Amino-acetyl BSA	0.5 amino	Acetamide	7.4	1.4	0.6
Methylene-tyrosine polymer	34 amino	Acetamide	1.2	3.3	
		Acetamide	6.2	9.6	10
		None	6.2	...	3
Lysozyme	5 amino	Acetamide	5.0[b,d]	4.7	
		Acetamide	7.4[b,e]	1.7	
Insulin	5 amino	Acetamide	4.0	2.2	
		Acetamide	7.0	1.5[e]	
Salmine sulfate	38 guanidyl	Acetamide	4.2	1	2
		None	4.2	...	1

[a] 100 mg. protein and acetamide in approximately 1 ml. 4% formaldehyde, 0.4–0.8 *M* acetate or phosphate buffers, or 1 *N* and 0.1 *N* HCl for the first two experiments; held at room temperature for two or three days. Control experiments showed that no amide was introduced if the formaldehyde was omitted. [b] 8% formaldehyde was used in these experiments. [c] This reaction mixture gelled after addition of the formaldehyde. [d] Lysozyme remained water-soluble under these conditions, whereas gelation occurred and an insoluble product was obtained in the absence of acetamide. [e] Insulin dissolved during the course of the reaction with acetamide at *p*H 7 but not at *p*H 4, nor with other additives (Table IX).

TABLE IX

FIXATION OF METHYLGUANIDINE SULFATE (MGS) BY MACROMOLECULAR COMPOUNDS CONTAINING AMINO GROUPS THROUGH FORMALDEHYDE CONDENSATION[a]

Macromolecular compound	Amino groups per 10^4 g.	Additive	Final *p*H	Equivalents bound per 10^4 g. Methylguanidine (Kjeldahl)	Equivalents bound per 10^4 g. Methylguanidine (Colorimetric)	Equivalents bound per 10^4 g. Formaldehyde
Bovine serum albumin (BSA)	9.7	MGS	2.7[b]	0	0.3	
		MGS	4.8	5.5	3.7	
		MGS	7.4[b]	5.5	3.3	
Amino-acetyl-BSA	0.5	MGS	4.8	0		
Methylene tyrosine polymer	34	MGS	3.2	4.5[c]		13
		MGS	4.7	24		60
		MGS	4.7	14[d]		29[d]
		MGS	8.5	23[c]		43
		None	6.2	...	...	3
β-Lactoglobulin	8.9	MGS	4.2	4.3	3.3	8
		MGS	7.6[e]	5.8	3.0	8
		None	7.6[e]	...	...	2
Insulin	5.0	MGS	4.0[f]		0.8	
		MGS	7.0[f]		1.3	

[a] Conditions and control experiments same as used in experiments listed on Table VIII (footnote *a*) but with methylguanidine sulfate instead of acetamide. For techniques and limitations of analytical methods see Experimental Part. [b] Gels form at *p*H 2.7 overnight, at *p*H 7.4 instantaneously upon addition of formaldehyde. [c] Residual amino nitrogen in those preparation, 22 and 5 equivalents, respectively. [d] Reaction time only five hours. [e] Reaction mixtures gel, then liquefy. [f] Protein did not dissolve in reaction mixture.

and N,N′-diphenyl-*p*-phenylenediamine led to rapid stable gelation; N-phenylglycine caused slow and non-permanent gelation. Hexamethylenetetramine acted somewhat more slowly than ammonia.

The gelling effect of ammonium chloride, which was studied in more detail, was not dependent upon the above conditions, but occurred also in more dilute acetic acid (10%) or formaldehyde (4%) solution, as well as in aqueous ethanol with or without added acetic acid, but not in alkaline alcoholic solutions. No gelling could be produced if aminonium chloride or an effective amine was added after the protein–formaldehyde reaction mixture had stood for three days. High concentrations of urea neither prevented gel formation nor redissolved the gel, once it had formed.

No definite information was available concerning the mechanism of these phenomena. A plausible explanation was suggested by the results of the model experiments discussed above. Both zein and gliadin contain a very great excess of amide over amino groups. For gliadin this ratio is about 40, in contrast to 1–2 for many typical proteins. Therefore little cross-linking between the two types of groups can occur in proteins like gliadin and zein. There is thus ample opportunity for small-molecular-weight amines to be bound through methylene bridges to the amide groups of the protein molecule. If these amines are bifunctional in nature, they may act as cross-linking agents between protein molecules, according to reaction V.

In agreement with this hypothesis, only primary

$$2R{-}CONH_2 + HCHO + R'NH_2 \longrightarrow RCONH{-}CH_2{-}NR'{-}CH_2{-}NH{-}COR + 2H_2O \quad (V)$$

amines or secondary diamines (*e. g.*, piperazine) are effective gelation catalysts.[25] When greater than "catalytic" amounts of the cross-linking amines were used, their introduction into the protein under the conditions of gelling was demonstrated analytically (Table VII).

The course of the gelation reaction is in accord with the results of the model experiments with acetamide and alanine. These have shown that, at *p*H 3–6, the cross-linking proceeds considerably faster than does the fixation of formaldehyde by the amide alone. In conformity, gliadin gels within a few minutes after addition of an effective amine. If excessive amounts of amine are avoided, many amide groups remain unaffected by the cross-linking reaction and, more slowly, are

(25) In confirming studies now in progress (footnote 5), it has been found that only those amines that cause gelation favor the formation of derivatives whose average molecular weight is increased over that of the proteins.

transformed into amido-methylol groups.[26] This probably contributes to liquefaction of the gels within twenty-four hours and explains the water solubility of the final products. Further, when alanine was used as the cross-linking agent, its fixation was found to be reversed during prolonged exposure to the 50% acetic acid–formalin reaction mixture. It appears probable, though it was not demonstrated, that ammonia may also be slowly released under such conditions. No re-solution was observed, however, if more extensive cross-linking had been produced through the addition of somewhat more of the amine (with ammonium chloride, 10–20% of the weight of the protein, instead of 1–2%). The gels then were stable and the products after isolation were insoluble in water, acetic acid, and 10 *M* urea solution.

The reaction mechanism postulated for the simple systems is also supported by the experiments with gliadin. Thus, if the amine or ammonia was added to the reaction mixture only after several days of standing, *i. e.*, after the amide groups had been transformed to amido-methylol groups, no gelation nor fixation of the amine occurred.

The behavior of gliadin (and zein[21,22]) is largely duplicated by that of polyglutamine, thus removing the possibility of protein groups other than the amide playing a role in this reaction. Under the same formaldehyde reaction conditions usually used with gliadin (50/50 glacial acetic acid and formalin), this polyamide, in 10% solution, gelled within thirty minutes if traces of ammonium chloride were present, but otherwise only after two to three days. Also, in 4–8% formaldehyde solutions containing as little as 3% of the polypeptide and 3% acetic acid, gels or precipitates were obtained only in the presence of small amounts of ammonium chloride or primary amines. As previously stated, the introduction of the amine or amino acid could be analytically demonstrated when greater than "catalytic" amounts were used (Table VII).

Discussion

The data presented show that the amino groups of proteins may readily condense with formaldehyde and amide or guanidyl groups under conditions of temperature and *p*H at which each type of compound, separately, binds no, or very little, formaldehyde in stable manner. The reaction of asparagine with formaldehyde represents an intramolecular condensation of this same type.[28]

Marvel, *et al.*,[29] have recently expressed the stimulating idea that urea in condensing and polymerizing with formaldehyde may act as an amino acid amide. On the basis of this hypothesis they studied the reaction of some amino acid amides with formaldehyde, and obtained polymers. In the light of the present findings, the primary reaction would appear to be a chain condensation of amino and amide through methylene groups. It does not appear probable that cyclic trimers are involved, as was suggested by these workers,[29] since the polymerization reaction proceeds only under conditions unfavorable for the formation, or even for the existence, of such trimers (Table IV, *cf.* footnote 14). Further, the amount of formaldehyde bound by the polymer was only slightly in excess of that equivalent to the glycine amide units, whereas 1.5 equivalents would be needed for the extensively cyclized and cross-linked structure postulated.[29]

In view of the many amino as well as amide and guanidine groups present in most proteins, and of the ease with which pairs of these can condense with formaldehyde, it appears probable that these reactions may play an important role both in the hardening and tanning action of formaldehyde and in its use in the preparation of vaccines and toxoids. The reaction further may supply a useful means of introducing a great variety of primary amines, amides, or guanidines stably into proteins. In the presence of an excess of a small molecular amide, the methylol-amino groups of the protein appear to condense preferentially with this instead of with protein amide groups. Thus cross-linking between protein groups may be largely forestalled and soluble derivatives obtained at a *p*H which favors the formation of insoluble coagula in the absence of the simple amide (Tables VIII and IX). On the other hand, bifunctional amines furnish cross-links in proteins deficient in amino but rich in amide groups, and bifunctional amides or guanidines may be expected to act similarly in proteins rich in amino but deficient in these types of groups.

Besides methylene condensations involving two nitrogenous groups, other reactions can occur which may contribute to the tanning action of formaldehyde. Mannich reactions involving aromatic and heterocyclic side chains and amino groups will be discussed in a subsequent publication. Similar reactions between basic groups, formaldehyde and reactive aliphatic methylene groups (*e. g.*, R—CH_2—COOH),[14] possibly account for the large amounts of formaldehyde bound irreversibly by proteins at elevated temperature.

(26) Proteins and model polymers rich in amide groups bind much formaldehyde (a) at elevated temperature in dilute acetic acid solution[27] and (b) at room temperature in alkaline solution (*p*H 11–12) (unpublished). The present data (Table VII) show that the reaction occurs at room temperature also at high formaldehyde concentration (20%) in 50% acetic acid, but not to a similar extent in more dilute solutions, unless amines or ammonium salts are present.

(27) Fraenkel-Conrat, Cooper and Olcott, THIS JOURNAL, **67**, 950 (1945).

(28) Levy and Silberman [*J. Biol. Chem.*, **116**, 723 (1937)] and others (*cf.* French and Edsall, footnote 17) have shown that a tetrahydropyrimidine ring is formed through condensation of formaldehyde with the amino and amide groups of asparagine. In contrast to Levy and Silberman's observations, we found that this reaction, like similar intermolecular condensations between amino acids and amides, was favored by a higher *p*H (*p*H 6.5 *vs.* 3.2) (*cf.* Fig. 1).

(29) Marvel, Elliott, Boettner and Yuska, THIS JOURNAL, **68**, 1681 (1946).

Experimental

Methods and Materials.—Free, plus labily bound, formaldehyde was determined by means of dimedon at *p*H 4.6[30]; total recoverable (*i. e.*, free and acid hydrolyzable) formaldehyde, by combined hydrolysis and distillation followed by colorimetric[31] or dimedon[30] analysis of the distillate. Irreversibly-bound formaldehyde was estimated by the difference between the amount originally added and that recoverable by acid hydrolysis from reaction mixtures. Other analytical methods used were the Kjeldahl procedure for total nitrogen, and the manometric Van Slyke[32] and colorimetric ninhydrin[33] methods for amino nitrogen. The Folin uric acid reagent was used for SH tests according to Anson.[34]

The introduction of amides into proteins was demonstrated by increases in amide-nitrogen content: 10–25 mg. of protein was autoclaved with 1.2 *N* sulfuric acid at 120 lb. for two hours; the solution was then neutralized, buffered with phosphate at *p*H 7.4, and the ammonia distilled off in a Kjeldahl apparatus. Recoveries of nitrogen from acetamide by this technique averaged 98.5%.

The introduction of methylguanidine led to appreciable increases in the non-dialyzable nitrogen, which served as an approximate measure of the extent of fixation of this compound. More specific and possibly more accurate were colorimetric analyses of the increases in chromogenic activity by the Sakaguchi method, as applied by Brand and Kassell.[35] These data, however, may be somewhat low, since indications were obtained that hydrolysis of methylol arginine peptides leads to a partial destruction of the chromogenic group.

The introduction of amines into proteins represented the greatest analytical problem. When a polyamide, such as polyglutamic acid, was treated with an excess of amine, sufficient amounts were introduced so that Kjeldahl analyses could be relied upon as proof for its fixation. However, when only 1–5 equivalents (per 10^4 g.) of amine were introduced into a protein, increments in the total nitrogen by about 1–3% could not be demonstrated with sufficient accuracy, particularly when the reaction was performed in acid solution and caused some protein degradation. Tyrosine was used in some experiments and its fixation was demonstrated colorimetrically; however, only one equivalent was bound by 10^4 g. gliadin (32 amide groups), probably because of the insolubility of tyrosine. The use of 2-aminoethylsulfuric acid proved more advantageous. Its introduction into gliadin was demonstrated by sulfate-sulfur analyses[36] and supported by nitrogen analyses. Finally, the acid groups introduced into proteins or polyglutamine through methylene condensation with amino acids were demonstrated by a dye method.[37] This method supplied the most clear-cut and trustworthy evidence concerning the relative non-reactivity of secondary, as compared to primary, amines (*viz.* amino acids).

The gliadin preparations were kindly furnished by D. K. Mecham; lysozyme, by G. Alderton and H. L. Fevold; β-lactoglobulin, by E. F. Jansen; edestin, by D. M. Greenberg; salmine sulfate and insulin, by the Eli Lilly Company. Polyglutamine was prepared as previously described.[27] Aminoacetyl serum albumin was prepared from commercial crystalline bovine serum albumin by acetylation with acetic anhydride in concentrated sodium acetate solution.[38]

(30) Yoe and Reid, *Ind. Eng. Chem., Anal. Ed.*, **13**, 238 (1941).
(31) MacFadyen, *J. Biol. Chem.*, **158**, 107 (1945).
(32) Van Slyke, *ibid.*, **8**, 425 (1929).
(33) Harding and MacLean, *ibid.*, **24**, 503 (1916).
(34) Anson, *J. Gen. Physiol.*, **25**, 355 (1941–42).
(35) Brand and Kassell, *J. Biol. Chem.*, **145**, 359 (1942).
(36) Mease, *J. Research Natl. Bur. Standards*, **13**, 617 (1934); *cf.* also 24.
(37) Fraenkel-Conrat and Cooper, *J. Biol. Chem.*, **154**, 239 (1944).
(38) Olcott and Fraenkel-Conrat, *Chem. Rev.*, **41**, 151 (1947).

Isolation of Amino Acid–Formaldehyde–Acetamide Condensation Products—N-(N-Acetamido-methylene)-alanine.—To 8.9 g. (0.1 mole) of alanine and 5.9 g. (0.1 mole) of acetamide dissolved in water, was added 30 ml. of commercial 40% formalin and water to 100 ml. After three days of standing at room temperature, 93 ml. of the solution was cooled and poured into a five-fold amount of chilled acetone. The precipitate was washed with acetone and dried *in vacuo* (9.4 g.) (59% yield). Recrystallization was effected by dissolving 1 g. in 13 ml. of ice water, and then adding 20 ml. of chilled ethanol. The compound decomposes above 180°.

Anal. Calcd. for $C_6H_{12}O_3N_2$: C, 45.0; H, 7.50; N, 17.5; HCHO, 18.7. Found: C, 44.9; H, 7.56; N, 17.5; HCHO, 18.4.

N-(N-Acetamidomethylene)-proline.—1.15 g. of proline and 0.59 g. of acetamide were dissolved in 2 ml. of 40% formalin. After two days at room temperature the reaction product was precipitated with acetone and repeatedly redissolved in a little water and reprecipitated with acetone. Crystallization occurred both in the oily, insoluble fraction and in the mother liquor; 700 mg. was isolated (38%). Partial decomposition of the product during recrystallization from cold water–acetone mixtures may account for the low recoveries (15% or less). But the unrecrystallized material appeared to be quite pure; m. p. 135–138° (dec.).

Anal. Calcd. for $C_8H_{14}O_3N_2$: C, 51.6; H, 7.43; N, 15.1; HCHO, 15.4. Found: C, 51.5; H, 7.55; N, 15.1; HCHO, 15.4.

Stability of Condensation Products.—The crystalline condensation products of alanine or proline with formaldehyde and acetamide were found to be comparatively stable in the presence of dimedon at *p*H 4.6. This made it possible to use the dimedon technique to determine the extent of hydrolysis that occurred in aqueous solutions of varying *p*H. The alanine derivative was also stable under the conditions used for Van Slyke manometric amino nitrogen analyses, so that the liberation of amino nitrogen could be used as an additional measure of the extent of hydrolysis. The data obtained by these methods are listed in Table X. It is evident that the stability optimum of the alanine derivative is more alkaline than that of the proline derivative.

TABLE X

ABILITY OF REACTION PRODUCTS IN AQUEOUS MEDIA

Medium, buffer and/or solutes	Conditions *p*H	Temp., °C.	Time, days	Extent of hydrolysis[a] of acetamide-formaldehyde condensation product of Alanine, %	Proline, %
0.2% Dimedon,	4.6	23	1	9	5
acetate	4.6	40	3	96	98
0.1 *M* Citric acid	2.0	23	1	36	18
0.5 *M* Acetic acid	3.4	23	1	28 (25)	14
Water	4.2	23	1	25 (16)	8
Phosphate	7.6	23	1	10 (14)	25
Borate	9.1	23	1	10 (10)	..
Phosphate	11.7	23	1	15 (8)	35
2.5 *M* Sodium nitrite in 2 *M* acetic acid (manometric Van Slyke)		23	3 min	6	
10% Pyridine (colorimetric ninhydrin)		100	30 min.	97	

[a] Figures in parentheses are based on Van Slyke manometric amino nitrogen analyses; all other figures are derived from dimedon analyses for liberated formaldehyde.

The finding that formaldehyde and amino nitrogen are liberated to a similar extent indicates that under the conditions used hydrolysis occurs first between the amide and methylene group—a true reversal of the two-stage reaction mechanism (Reaction IV, followed by III). A primary breaking of the amino-methylene linkage would yield methylol acetamide which would not liberate much formaldehyde at room temperature and *p*H 3.4. (N-Methylol-acetamide was hydrolyzed to only 2.4% in twenty-four hours under such conditions.)

Failure to Achieve Condensation of Amido-methylol with Amino Groups.—In the hypothetical reaction

$$R{-}CONH{-}CH_2OH + R'{-}NH_2 \longrightarrow R{-}CONH{-}CH_2{-}NH{-}R' + H_2O$$

there would be no fixation of formaldehyde, but measurable loss in amino nitrogen. In one experiment, the amido-methylol compound was formed by adding 0.1 ml. of 0.1 *N* sodium hydroxide to a solution of 0.5 mM. of acetamide in 1 ml. of 8% formaldehyde (2.5 mM.). After one hour at room temperature, the solution was neutralized with 0.1 ml. of 0.1 *N* hydrochloric acid, buffered at approximately *p*H 3.4 with 0.2 ml. of 3 *N* acetic acid, and then to it was added 0.5 mM. of alanine. After three days of standing at room temperature, the mixture was diluted and analyzed. The reaction of the amide with formaldehyde approached completion (0.47 mM.), as expected,[11] but there was no loss in amino nitrogen, *i. e.*, no cross-linking had occurred with the alanine.

In another experiment N-methylolacetamide was isolated prior to the treatment with alanine[11]; 0.48 mM. of the oily preparation was mixed with 0.5 mM. of alanine and 1.25 ml. of 0.6 *M* acetic acid. A similar sample was made up to contain also 0.5 mM. of free formaldehyde. Neither sample, after a three day reaction period and dilution, showed a loss in amino nitrogen. Thus no cross-linking occurred between amido-methylol and amino or amino-methylol groups.

Changes in Optical Rotation of Amino Acid–Formaldehyde Solutions with and without Added Acetamide at *p*H 3–4. A.—L-Proline (2% solution) in 0.72 *M* acetic acid, $[\alpha]^{25}D$ −83°.

Upon addition of 3 ml. of 40% formaldehyde to 12 ml. of this solution, the *p*H dropped from 3.0 to 2.9 in one hour, then remained constant. $[\alpha]^{25}D$ after ten minutes, two and one-half hours and twenty hours, was −92, −94 and −94°, respectively.

Addition of 3 ml. of 40% formaldehyde to 12 ml. of 2% L-proline solution (as above) containing also 1.04% acetamide (one equivalent) caused the rotation to rise to −91.5° after five to one hundred and twenty minutes, but to drop within 19, 43 and 67 hours to −85, −80.5 and −78°.

B.—L-Leucine (2% solution in 0.72 *M* acetic acid), $[\alpha]^{25}D$ −6.2.

After addition of 3 ml. of 40% formaldehyde to 17 ml. of amino acid solution, $[\alpha]^{25}D$ was −4.2, −3.5 and −3.0°, after ten minutes, three hours and twenty-one hours, respectively.

C.—L-Cystine (1% solution in 0.36 *N* hydrochloric acid containing 1% glycine, *p*H 0.85), $[\alpha]^{25}D$ −230°.

After addition of 3 ml. of 40% formaldehyde to 12 ml. of above solution, $[\alpha]^{25}D$ −212 to −214° after five to one hundred and eighty minutes.

Addition of amounts of acetamide to the formaldehyde-containing solution, equivalent to the sum of the amino acids present, caused a change of rotation within one hour to $[\alpha]^{25}D$ −180°, and after three, sixteen and forty hours to −182, −190 and −194°, respectively.

Attempts to Demonstrate Condensation between Alanine, Formaldehyde, and Acetamide in Strongly Acid and Alkali Solution.—The change in rotation noted in the case of the L-cystine–formaldehyde reaction mixture of *p*H 0.85 when acetamide was added suggested that interaction of the amino-methylol and amide had occurred. It was attempted to establish this more firmly in a simpler system by using Van Slyke amino-nitrogen analyses on alanine–acetamide–formaldehyde reaction mixtures of similar *p*H. Parallel experiments were also performed in alkaline solution (*p*H 11).

At both *p*H levels, acetamide alone binds formaldehyde readily, so that the aldehyde analyses could not be used as evidence for cross-linking. Amino-nitrogen analyses, performed after dilution and neutralization, gave high and erratic results until the free and dimedon-reversibly bound formaldehyde was removed from the solution (by means of dimedon). The presence of ammonia, formed through incipient hydrolysis of the acetamide, detracted from the quantitative significance of the data.

One typical experiment is shown in Table III. It appeared that there is little, if any, cross-linking in acid solution in twenty-four hours, and little cross-linking at *p*H 11. The (irreversible) fixation of formaldehyde and loss of amino nitrogen of alanine alone under the latter conditions interferes with quantitative interpretation of the amino-nitrogen data on the alanine–acetamide reaction mixture.

Search for Methylene-Condensation Reactions Involving Thiols and Guanidines.—When thioglycol reacted with formaldehyde over the range of *p*H 4.3–7.6 with or without added alanine or acetamide, no more formaldehyde was bound than could be accounted for by the added nitrogenous compound, and no loss in chromogenic activity of the thiol occurred (Table XI). This is regarded as evidence that no R–S–CH_2–NH-R bonds were formed unless these were so labile as to be hydrolyzed during dilution and analysis (*cf.* footnote 13).

TABLE XI

FIXATION OF FORMALDEHYDE IN REACTION MIXTURES CONTAINING ALANINE OR ACETAMIDE WITH THIOGLYCOL OR METHYLGUANIDINE

Reactants	Final *p*H	Time, days	Formaldehyde bound, equivalents[a]	% Thiol disappeared
Alanine (1)[b] + thioglycol (1)	7.6	4	0.18	0
Alanine (1) + thioglycol (1)	4.6	3	.09	0
Alanine (1) + thioglycol (1)	1.4	3	.47	97
Alanine (1)	7.6	1	.22	..
Alanine (2)	4.3	4	.10	..
Alanine (1)	1.4	1	.00	..
Thioglycol (2)	7.6	4	.00	0
Thioglycol (2)	4.3	4	.00	
Thioglycol (2)	1.4	3	.50	99
Acetamide (1) + thioglycol (1)	7.6	4	.18	
	4.3	4	.08	
Acetamide (2)	4.3	4	.22	
Alanine (1) + methylguanidine (1)	4.3	4	1.34	
Methylguanidine (2)	4.3	4	1.26	
Methylguanidine (1)	4.3	4	0.65	
Acetamide (1) + methylguanidine (1)	4.3	4	0.75	
Alanine (0.5) + methylguanidine (0.5)	4.7	4	1.34	
Methylguanidine (0.5)	4.7	4	0.46	
Alanine (0.5)	4.8	4	0.10	

[a] In terms of alanine if present; otherwise in terms of the potential reactant present. [b] Figures in parentheses indicate millimoles used (in 2.5 ml. of reaction mixture containing usually 4–5 millimoles of formaldehyde).

At *p*H 1.5, the disappearance of one-half equivalent of formaldehyde for one of thioglycol suggests that it was transformed quantitatively to the methylene bis-(ethanol) thioether, (CH_2OH–$(CH)_2$–S–CH_2–S–$(CH_2)_2$–CH_2OH), both in the presence and the absence of alanine

(Table XI); the compound was not isolated. Armstrong and du Vigneaud[39] have recently described a method of synthesis of djenkolic acid from cysteine in strong hydrochloric acid, which represents an analogous reaction.

Methylguanidine sulfate binds formaldehyde by itself in solutions above *p*H 7 and below *p*H 5. However, much more formaldehyde is bound at *p*H 4.7 and 4.3 in the presence of alanine than in the presence of acetamide or in solutions of methylguanidine sulfate alone, which is regarded as evidence for cross-linking between guanidine and amino groups (Table XI). The formation of such cross-links was further indicated by the fixation, in the presence of formaldehyde, of methylguanidine residues by proteins or model substances rich in amino groups (Table IX).

Little, if any, cross-linking between guanidine and amide groups appears to occur in the experiments listed in Tables VII, VIII, and XI. This is borne out by a preparative experiment with methylguanidine sulfate. A mixture of 5 millimole each of this compound and acetamide in 2 ml. of 40% formalin was held at room temperature for three days and at 53° for three hours, then isolated and washed by repeated acetone precipitation. The final product contained 92% of the methylguanidine nitrogen and only approximately 0.1% amide of total N.

Polymerization of Glycine Amide with Formaldehyde.[29]—To 110.5 mg. of glycine amide hydrochloride (1 mM.), dissolved in 1 ml. of 3 *M* sodium acetate, was added 0.6 ml. of 7.5% formaldehyde (1.5 mM.). The reaction mixture solidified to a white gel within twenty-four hours. After two days of standing, the product was isolated by repeated cycles of trituration with water and centrifugation. The last washing was free from formaldehyde. The supernatant and washings were pooled; the insoluble polymer was dried (over sodium hydroxide flakes) from the frozen state.

The pooled solution (50 ml.) was at *p*H 4.8. It contained 0.435 mM. of glycine amide (by nitrogen analysis), and 0.525 mM. free formaldehyde (dimedon). The total formaldehyde in the solution represented 0.885 mM. From these results it may be calculated that the insoluble polymer contained 0.565 mM. glycine amide and 0.615 mM. of formaldehyde, *i. e.*, 1.09 equivalents of formaldehyde per glycine amide unit. Very similar results were obtained in two further experiments in one of which 2.0 equivalents of formaldehyde were used. In all experiments, the nitrogen recovery in the two fractions was only 96–97% (formaldehyde recovery 99–100%), and the reproducibility of Kjeldahl analyses on the polymer was poor. Dumas analyses gave a lower value (25.1%). C and H analyses performed on a preparation that had been dried at 100° in high vacuum and then permitted to equilibrate with laboratory air, were difficult to interpret because of the probable absorption of carbon dioxide by the basic polymer (C, 38.3; H, 6.86; N (Kjeldahl), 25.3–26.6; HCHO, 31.7; weight loss and regain, 12.1%). The dried polymer contained no acetate. It was insoluble in all solvents tried, including saturated lithium iodide, and 1,3-dichloropropanol, solvents for silk fibroin, and nylon, respectively.

Use of Cyclic Trimers (—NR—CH_2—)$_3$ in Condensation Reaction.—Cyclic trimers were prepared from formaldehyde and both methylamine and *n*-butylamine. When these compounds were held at *p*H 4 or 5 in the presence of acetamide, much formaldehyde was bound stably (to dimedon at *p*H 4.6), while in the absence of acetamide almost the entire formaldehyde was liberated and could be precipitated with dimedon (Table IV). When the trimer was held at *p*H 8.3, no interaction occurred with acetamide, and all formaldehyde could be precipitated with dimedon after dilution and one day of exposure to *p*H 4.6 acetate (*cf.* footnote 14).

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Summary

Simple primary and secondary amines react with formaldehyde and primary amides over the range of *p*H 3–8 within twenty-four to forty-eight hours at room temperature to give condensation products of the general structure: R—NR′—CH_2—NH—CO—R″. Representative compounds of this type have been isolated in pure form.

Evidence is presented that the primary reaction is the formation of methylol-amines, not methylol-amides.

N-Alkyl amides, including peptides (RNH—CO—R′), do not react in similar systems.

Simple amines also condense with formaldehyde and guanidines at *p*H 4–5, but amides and guanidines do not react under the same conditions.

Through methylene condensation reactions of these types, formaldehyde permits the introduction of simple amides or guanidines into proteins or macromolecular model substances rich in amino groups, and also permits the introduction of primary amines into proteins or model substances that are rich in amide groups.

Under suitable conditions, small amounts of ammonia or primary amines cause gelation of acid formaldehyde reaction mixtures of gliadin, zein, or polyglutamine, probably by introducing cross-links $\left(-CH_2-\underset{\displaystyle R}{N}-CH_2-\right)$ between the numerous amide groups of these substances.

While not unequivocally demonstrated, it appears very probable that these cross-linking reactions between amino and primary amide or guanidyl groups contribute greatly to the tanning or hardening action of formaldehyde at room temperature and over the range of *p*H 2–9, on proteins of average composition.

(39) Armstrong and du Vigneaud, *J. Biol. Chem.*, 168, 373 (1947).

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